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Observations upon the Application of Partition Chromatography to the Determination of the Monoamino-acids in Proteins

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Synge (1939) and Martin & Synge (1941*b*) have shown that the acetamino-acids

have characteristic partition coefficients between organic solvent and water. As a result of experience gained (Martin & Synge, 1941*a*) a new form of chromatography was introduced (Martin & Synge, 1941*b*) which employs two liquid phases. The stationary phase (water) is held by a carrier (silica gel) which under ideal conditions should not adsorb the acetamino-acids. (Ryan & Brand (1944) were in error when they described the method as adsorption chromatography.) The movement of the acid bands is marked by the presence of a suitable indicator in the aqueous phase. It has been shown that each acetamino-acid will, for a particular solvent mixture, move down the column at a definite rate (see Gordon, Martin & Synge, 1943*a*) although evidence has been obtained in the present work which suggests that the *R* value (i.e. movement of band/movement of surface) may be influenced by the presence of other acetamino-acids upon the column and by the water content of the silica.

In succeeding publications the Leeds group (Gordon *et al.* 1943*a*, 1944) have improved the method and extended it to include phenyl-alanine, the leucine isomers (leucine and *isoleucine* cannot

be resolved) proline, valine, tyrosine and alanine. They also included methionine, but evidence will be presented in the present paper which suggests that its recovery is dependent upon the particular amino-acid mixture analyzed and possibly upon the nature of the silica gel.

Partition chromatography was the first method which made possible the serial micro-estimation of the above group of amino-acids. It has been suggested (Martin & Synge, 1945) that the ultimate aim of protein analysts should be the adoption of a routine procedure applicable to small quantities of protein, and realizable, as Chibnall (1946) has pointed out, only by assessing the relative merits of the various methods of analysis. It is with this object in view that considerable prominence has been given to a discussion of the comparative data so far available. Certain recommendations have also been made which it is hoped will facilitate future comparative analyses.

Apart from preliminary experiments on gelatin and wool (Gordon *et al.* 1943*a*, 1943*b*) the partition method has not been used for the analysis of the more complex proteins. The author has made extensive use of it for the analysis of many proteins, and the procedure as suggested by the Leeds workers has not been modified except in minor details. Certain anomalous results have been obtained, however, and suggestions are made to overcome such difficulties that may arise.

EXPERIMENTAL

Reagents used. 1. Acetic anhydride. The analytical reagent should be distilled before use (20 cm. packed column) to remove acetic acid and non-volatile impurities which may vitiate the acetylation.

4. *Cyclohexane*. The laboratory reagent contains benzene in variable amounts; it was not found necessary however to remove this before use.

5. Chloroform. All grades of reagent were washed free from ethanol and distilled before use.

6. Indicators. (a) Methyl orange (henceforward referred to as M.O.). The solution used was 0.015% in aqueous

Diagram 1

Diagram 2

Diagram 3

Numbers on diagrams indicate the fractionation sequence (see Gordon *et al.* 1943*a*).

Code	Solvent mixture (v/v) (saturated with water)	Code used in text	Indicator
*	CHCl ₃ -17% butanol	CB ₁₇	ARR
†	CHCl ₃ -2% <i>n</i> -butanol	CB ₂	M.O.
‡	Ethyl acetate	Et _{ac}	ARR
§	Cyclohexane-5% <i>n</i> -propanol	P ₅	"
	Cyclohexane-30% <i>n</i> -propanol	P ₃₀	"

2. *n*-Butanol. The reagent grade of solvent may contain Ca⁺⁺. It should be distilled (40 cm. packed column) and the fraction boiling at 116–117° (760 mm.) collected for use.

3. *n*-Propanol. The technical solvent is reasonably pure and should be fractionally distilled b.p. 97° before use.

solution. (b) *Alphamine Red-R* (referred to henceforward as ARR). Described by Liddell & Rydon (1944) under the name Indicator *R*-NH₄. These workers have agreed to, and are responsible for, the change of name to facilitate abbreviation.

Hydrolysis of the proteins. The proteins were hydrolyzed by the procedure adopted as standard by Chibnall and his co-workers, viz. protein was allowed to dissolve in 5 vol. conc. HCl (A.R. Δ 1-18) at 37°. The solution was then heated under reflux on a boiling water-bath until frothing ceased. The acid was then diluted to constant boiling mixture (5-5N) by the addition of water, and hydrolysis brought to completion by boiling under reflux for 24 hr. Excess acid was removed by repeated concentration *in vacuo* (bath at 45°), the amino-acid solution made to volume and samples removed for analysis.

Preparation of the amino-acids for analysis. The sample was adjusted to 2-3 ml. and the amino-acids acetylated by the method of Gordon *et al.* (1943a). The acetamino-acids were extracted from the acetylation mixture by the chromatographic procedure (Gordon *et al.* 1943c). In preliminary experiments it was found that small amounts of H_2SO_4 , sufficient to cause esterification and decomposition, were being extracted by the eluting solvent. This was overcome by introducing a pad of gel saturated with water (5 g. of gel will give a pad 10 mm. in thickness in a column 4 cm. in diameter). In general this effected the removal of H_2SO_4 but the following additional precaution was taken: 5 ml. of water were added to the receiver at the beginning of the elution and a sample (1 ml.) of the aqueous solution, withdrawn when elution was complete, was tested for sulphate. If sulphate was present the aqueous layer was removed in a separating funnel, and washed several times with the eluting solvent, all the organic solvent being filtered through a No. 2 Whatman filter paper.

The Leeds workers have stressed the need for acidifying the acetylate to pH 1.0 to ensure the complete extraction of the acetamino-acids. This has been confirmed in the present work. If the acetylate was acidified to pH 3.0 for instance, the recovery of phenylalanine was quantitative, but the recovery of valine and glycine was 30 and 0% respectively.

The complete removal of ethanol (used in the transference of the acetamino-acids to a small flask), butanol and acetic acid, is extremely important, and the following drying procedure has been adopted. The bulk of the ethanol is removed *in vacuo* (bath at 60°) and the residue then dried overnight in a vacuum desiccator at 0.005 mm. The drying agents used were anhydrous $CaCl_2$ and pellet KOH; the use of H_2SO_4 as suggested by the Leeds workers is not favoured.

Fractionation procedure. Gordon *et al.* (1943a) suggested a scheme for the fractionation of the amino-acids (Diagram 1). This procedure was used during the early part of this work, which was mainly concerned with insulin, and the low and variable recoveries of proline from insulin hydrolysates and their attendant control solutions (60-75%) were assumed to be due to the small amount of proline involved. The satisfactory recovery of somewhat larger amounts of proline from edestin and casein controls suggested that this was indeed the case, but later analyses on lactoglobulin controls, in which the recovery of proline was consistently low, made it clear that the procedure was not capable of universal application. An alternative procedure (Diagram 2) was tested and found to give satisfactory recoveries of proline from control solutions representing insulin (95-99%) and lactoglobulin (99.4%) hydrolysates. A further procedure (Diagram 3) was found to give rather unexpected results which will be discussed later.

The preparation of silica gel

The preparation of satisfactory samples of silica gel was shown by Gordon *et al.* (1944) to be difficult and has been the main problem encountered in the present work. As mentioned above, the ideal gel should not adsorb the acetamino-acids, and it was observed by the Leeds group that, from this point of view, the properties of any given sample of gel appeared to depend upon certain unspecified properties of the waterglass, the temperature of precipitation and, in certain instances, upon the process of 'ageing'. These observations have been confirmed in the present work. It has also been observed that the dilution of the waterglass with two vol. of water, recommended by the above workers, is an important factor. More recently it has been noted (Lindley, 1946, and the present work) that any adsorption of acetamino-acids which does occur is a function of the water content of the gel. Since the gels used in this laboratory have been prepared by the method of Gordon *et al.* (1943a) or by simple variations of their procedure, it is proposed to limit the discussion given below to those factors which appear to exert a controlling influence on the properties of the gel.

The purity of the waterglass. It has been observed that the properties of silica gel obtained from any one batch of waterglass tend to be independent of variations in the method of preparation. The reasons for the apparent dependence of the properties of the silica gel upon the waterglass used were not clear, although it was suspected that it was due to the presence of impurities (Fe, etc.) and to the state of aggregation of the silica molecules. It has now been shown (Islerwood, 1946) that Fe^{+++} and Al^{+++} , if present in silica, interfere with the estimation of organic acids, and that the aggregation of the silica molecules is also important since silica prepared from silicon halides may be highly adsorbent unless it is first aged by standing in HCl and also in the dry state for 21 days. It is of interest to note that the original batch of waterglass used by Martin and Synge in the development of the method contained only 0.027% Fe_2O_3 .

The temperature of precipitation. The silica gel should be granular to facilitate the drainage of the columns and should hold an optimal amount of water without losing its granular nature. It has been found that the particle size of the gel decreases as the temperature of precipitation increases. At the same time the resultant gels show a corresponding increase in adsorption of acetamino-acids. A convenient method of dilution at a temperature below 8° is as follows: the silicate is mixed with one vol. of water and cooled to 0°; the second vol. of water is then added in the form of crushed ice immediately before the precipitation with acid. When the thick stage of the precipitation is reached several volume equivalents of ice may be added without ill effect.

The concentration of the silicate. The dilution with two vol. of water appears to be highly critical for the preparation of a silica gel with the required properties. The effect of increasing the dilution is similar to, although more pronounced than, the effect of increasing the temperature. Thus the precipitates obtained from a solution diluted with four vol. of water tend to assume a gelatinous nature, while with five vol. the precipitates are translucent jellies.

'Ageing' of the silica. The need for 'ageing' or otherwise is largely determined by the waterglass used. Thus the γ gel

reported by Gordon *et al.* (1944) was not 'aged' and Dr H. Lindley (private communication) has recently prepared gels which did not require treatment with dilute acid. In the present work, however, 'unaged' gels have been invariably alkaline and 'ageing' by treatment with 0.05–0.1 N-HCl for 12–24 hr. has usually been necessary. If this latter treatment be prolonged, it may lead to an increase in the undesirable properties of the gel.

The water content of silica gel. The Leeds group suggested that silica gel prepared by their procedure reached saturation when about 53% (w/w) of water was absorbed on the surface of the gel. They recommended that the actual amount should be determined for each sample of gel. Saturation tests carried out, in the recognized manner, in this laboratory invariably agreed with the amount stated

Gordon *et al.* (1944) have suggested that each batch of gel be submitted to the following standard test: 'A mixture containing 3 mg. each of acetylphenylalanine and acetyl-leucine is subjected to analysis on a column (1 cm. diam.) containing 3 g. of the gel under test with CB₁, CB₂ or CB₃ as the developing solvent and M.O. as the indicator. The sum of the values should agree within 2% of the theoretical and the recovery of acetylphenylalanine should be at least 99%.'

This test was used in the present work until it was found that certain samples of gel, apparently normal according to the test, gave unsatisfactory results when a more complex mixture of acetamino-acids was employed. The results with one such gel (A) are recorded in Table 1 together with the recoveries when a normal gel (B) was used.

Table 1. *The determination of the efficiency of silica gel*

- (a) The efficiency test of Gordon *et al.* (1944).
(b) The efficiency test used in the present work.

Acid	Wt. taken (mg.)	Recovery (%)			
		Gel A exhibiting specific adsorption		Gel B with normal properties	
		(a)	(b)	(a)	(b)
Acetylphenylalanine	3.2	102, 98	100, 102	99, 98	103, 99
Acetyl-leucine	3.1	97.5, 101	98, 97	102, 101	97, 99
Acetylvaline	2.8	—	77, 79	—	96, 96
Acetylproline	2.7	—	64, 83	—	96, 97
N-Acetyltyrosine	2.9	—	94, 101	—	98, 98
Acetylalanine	3.1	—	82, 87	—	98, 99
Acetyl-glycine	3.0	—	65	—	96, 95

above. Lindley (1946), however, has recently noted that a sample of gel would absorb 75% of its weight of water. With this amount of water the recovery of acetamino-acids was normal. If, however, the water content of the acetamino-acids was reduced to 53% the recovery of acetamino-acids was irregular. Experiments in this laboratory confirmed this finding and indicated that little reliance could be placed on saturation tests. Three samples of a gel were placed in a saturated atmosphere, the original water contents being adjusted to 5, 50 and 75%. At the end of 14 days the water contents were 10, 50 and 74% respectively. The gels were then allowed to stand in the laboratory, when the water contents fell to 7, 30 and 35% respectively in 5 days. Three further samples of the gel containing 50, 75 and 85% water were then tested out with acetamino-acids. The recovery was irregular in the first case and normal in the remaining two samples, although the one containing 85% water showed a tendency to crack when left without a head of solvent. With a gel containing 50% water the *R* value of acetylalanine increased to 0.45–0.5, but with gels containing 70% water the value was normal (0.35). A sample of gel known to be highly adsorbent was next tried and showed only slight improvement with increasing water content. It was found to contain considerable amounts of Fe⁺⁺⁺.

It would appear from these experiments that, for a gel free from iron, the water content must be increased to an optimal point at which adsorption is reduced to a minimum while the silica still retains its properties as a powder. It has been found that the recovery of acetamino-acids from gels prepared by the method of Isherwood (1946) is also dependent upon the water content.

The standard procedure now in use is to subject a representative mixture of acetamino-acids to analysis and to reject all gels which give recoveries for each constituent of less than 95%.

Gels prepared by the procedure of Isherwood (1946). This worker has radically changed the method of preparation by making use of 10N-HCl for the removal of impurities (Fe and Al). The washed gel is then dried with ethanol and ether and allowed to stand for 21 days when the treatment with HCl is repeated. The use of large excess of HCl was tried in this laboratory and the resultant gels were highly adsorbent and extremely acid, which suggests that the period of standing is probably a critical factor. A gel prepared by Isherwood was tested and, although very acid, gave good recoveries from a simple mixture of acetylphenylalanine, acetylproline and acetylalanine, but when a mixture representing a complete hydrolysate was used, the recoveries were similar to those obtained with a gel prepared by the normal method, with certain isolated values erratic. Isherwood suggests that the gels prepared by his technique should be saturated with 100% of water. It was found that in this condition they are liable to crack and channel and that the indicators are not so firmly bound to the silica. With Solvent CB₁₇ for instance, considerable amounts of AR_R appeared in the filtrates.

The preparation of control mixtures

It is generally thought that the destruction or modification of certain amino-acids occurs more readily at the time of their actual liberation from the peptide chain than when they are present as the free amino-acids. With this limitation

the use of control mixtures can be of great value in demonstrating the accuracy and specificity of analysis and in aiding the assessment of the losses of those amino-acids (e.g. serine, tryptophan) which are known to be unstable in hot acid solutions. In addition it is possible, when using control solutions representing complete protein hydrolysates, to assess the effect, if any, of the amino-acid mixture on the estimation of any one particular amino-acid.

The compositions of the control mixtures (Table 2) used in this work, and by Rees (1946), are based mainly upon analyses carried out in this laboratory; certain of the monoamino-acid values used were taken from preliminary experiments and may differ slightly from those finally quoted. What appeared to be the most reliable value in the literature was used when no such analyses were available.

RESULTS OF ANALYSIS

The results obtained in the present work have been classified into two groups, (1) those in which the analysis is based on the recoveries obtained with an *ad hoc* control mixture (Table 4) and (2) those for which no *ad hoc* control analysis has been made (Table 6), where the recoveries are based on the mean values found for the four control mixtures used for the proteins in group 1. Needless to say the results given in Table 6 must be regarded as provisional, especially those for zein, gelatin and

Table 2. *Composition of the ad hoc control mixtures*

A. mg. of amino-acid or salt in the mixture which represents 1 g. of the protein concerned.

B. N as % total protein-N represented.

Amino-acid	Insulin		Edestin		Casein		Lactoglobulin		Horse haemoglobin	
	(A)	(B)	(A)	(B)	(A)	(B)	(A)	(B)	(A)	(B)
<i>dl</i> -Phenylalanine	80.9	4.41	59.5	2.71	64.5	3.5	43.0	2.34	79.0	4.0
<i>dl</i> -Leucine	109.0	7.5 ⁽¹⁾	85.5	4.89 ⁽¹⁾	105.5	7.2 ⁽¹⁾	162.0	10.68 ⁽²⁾	160.0	10.0
<i>dl</i> -Isoleucine	47.5	3.25 ⁽¹⁾	35.0	2.0 ⁽¹⁾	53.0	3.6 ⁽¹⁾	83.5	5.75 ⁽²⁾		
<i>dl</i> -Valine	75.0	5.76	47.6	3.05	60.0	4.6	54.0	4.15	79.5	5.65
<i>dl</i> -Methionine	0.0	0.0	23.4	1.18	35.0	2.1 ⁽¹¹⁾	32.0	1.94	9.5	0.53
<i>l</i> (-)-Proline	25.5	2.0	35.5	2.32	105.5	8.2	55.0	4.28	39.0	2.8
<i>l</i> (-)-Tyrosine	131.0	6.49	43.5	1.80 ⁽¹⁰⁾	63.0	3.1 ⁽¹⁰⁾	38.0	1.87	52.0	2.4
<i>dl</i> -Alanine	46.0	4.66	43.0	3.64	32.0	3.2	65.0	6.56	74.0	6.9
Glycine	43.0	5.16	38.0	3.80 ⁽⁴⁾	4.2	0.5 ⁽⁷⁾	19.0	1.59	—	—
<i>dl</i> -Tryptophan	0.0	0.0	15.0	1.09	13.7	1.2 ⁽⁹⁾	19.5	1.71	22.0	1.8
<i>l</i> (-)-Cystine	125.0	9.36	14.5	0.90	4.1	0.3 ⁽¹¹⁾	34.0	2.53 ⁽⁶⁾	10.2	0.71 ⁽¹²⁾
<i>l</i> (-)-Hydroxyproline	0.0	0.0 ⁽⁸⁾	20.0	1.15 ⁽⁴⁾	22.0	1.5 ⁽⁶⁾	0.0	0.0 ⁽²⁾	—	—
<i>dl</i> -Serine	52.0	4.48	63.0	4.51	59.0	5.0	39.0	3.34	58.0	4.60
<i>dl</i> -Threonine	21.0	1.57	38.5	2.43	45.5	3.4	51.0	3.85	43.6	3.05
<i>l</i> (+)-Glutamic acid (HCl)	232.0	11.39	257.0	10.54	275.0	13.4 ⁽⁶⁾	269.0	13.15	148.5	6.75 ⁽⁶⁾
<i>dl</i> -Aspartic acid	57.0	3.84	120.0	6.77	67.0	4.5 ⁽⁶⁾	99.0	6.66	148.5	9.32 ⁽⁶⁾
<i>dl</i> -Lysine (HCl)	31.0	3.01	29.6	2.44	103.0	10.1	140.0	13.8	96.0	9.4
<i>l</i> (-)-Histidine	49.0	8.50	29.0	4.2	32.5	5.6	15.5	2.68	79.0	12.7
<i>l</i> (+)-Arginine (HCl)	36.0	6.2	202.0	28.85	49.0	8.3	35.0	5.95	44.0	6.95
Amide ((NH ₄) ₂ SO ₄)	65.4	8.9 ⁽⁹⁾	83.5	9.49 ⁽⁹⁾	67.0	9.1 ⁽⁹⁾	50.0	6.85 ⁽⁹⁾	37.0	4.66 ⁽⁹⁾
As % total protein-N	96.48		97.76		98.4		99.68		92.22	

(1) Leucine-isoleucine ratios determined by Dr Sutherland (private communication).

(2) Brand, Saidell, Goldwater, Kassell & Ryan (1945).

(3) Dr Gordon from paper chromatograms (private communication).

(4) Abderhalden (1903).

(5) Fürth & Minnibeck (1932).

(6) Includes 0.82% cysteine-N (11 mg. cysteine).

(7) Williamson (1944).

(8) Bailey, Chibnall, Rees & Williams (1943).

(9) Chibnall (1942).

(10) Lugg (1938).

(11) Ryan & Brand (1944).

(12) Kuhn, Burkofor & Quackenbush (1939).

In the case of the control mixture representing horse carboxyhaemoglobin the monoamino-acid composition is based upon experiments now known to have been somewhat unsatisfactory (the silica gel in use at the time was later shown to be adsorbent) and the values quoted should be regarded as only approximate.

All the amino-acids were checked for purity by analysis and in most instances by the use of paper chromatography (Consden, Gordon & Martin, 1944). The protein samples used were those recorded by Macpherson (1946).

collagen, in which the amino-acid composition is widely different from that of any of the proteins of group 1. Chibnall (1946) reported values for certain of the proteins which may differ slightly from the final values recorded in Table 4. The modified values are due to the incorporation of additional results. The additional experience has not borne out the earlier claim (Chibnall, 1946) that the method was generally accurate to $\pm 2\%$.

Table 3. *Recovery of the monoamino-acids from control mixtures after 'hydrolysis'*

(Figures in parenthesis indicate the number of 'hydrolyses' carried out.)

- A. mg. N taken (see Table 1).
 B. mg. N recovered, each value being an independent acetylation.
 C. Mean recovery with standard deviation.
 D. Mean recovery as % (C/A).

	Phenylalanine	Leucine(s)	Valine	Proline	Tyrosine	Alanine
Insulin (5)						
A	0.172	0.418	0.224	0.078	0.253	0.181
B	0.163, 0.184, 0.160	0.429, 0.391	0.209, 0.218	(0.052)*	0.239, 0.238	0.192, 0.174
B	0.162, 0.165, 0.172	0.433, 0.433	0.219, 0.210	0.771, 0.741	0.261, 0.239	0.172, 0.188
B	0.165, 0.172	0.418, 0.429, 0.391	0.220, 0.208	0.771, 0.78	0.263, 0.253	0.181, 0.181
C	0.168±0.007	0.418±0.017	0.214±0.005	0.0766±0.015	0.249±0.011	0.181±0.007
D	97.8±4	100±4	95.5±2.4	98.3±2	98.5±4.5	100±4
Edestin (1)						
A	0.126	0.32	0.142	0.108	0.084	0.17
B	0.125, 0.136, 0.132	0.32, 0.291, 0.294	0.129, 0.132, 0.142	0.098*, 0.101	0.084, 0.08, 0.079	0.168, 0.167, 0.167
C	0.131±0.005	0.302±0.013	0.134±0.006	0.099±0.0015	0.081±0.002	0.167±0.0002
D	104±3.8	94.4±4.3	94.5±4.5	92.7±1.5	96.5±2.5	98.6±0.1
Lactoglobulin (1)						
A	0.091	0.655	0.162	0.167	0.073	0.256
B	0.091, 0.096, 0.091	0.663, 0.619, 0.648	0.155, 0.151, 0.159	(0.133)*, 0.160, 0.170, 0.167	0.073, 0.071, 0.070	0.252, 0.256, 0.254
C	0.093±0.002	0.64±0.02	0.155±0.004	0.166±0.004	0.071±0.002	0.254±0.002
D	102.2±2	98.2±3	95.8±2.5	99.3±2.4	97.3±2.8	99.3±0.8
Casein (1)†						
A	0.137	0.424	0.180	0.321	0.121	0.125
B	0.139	0.396	0.174	0.305, * 0.305	0.121	0.125
D	101.5	93.3	96.3	95.0	100	100
Mean of all controls:						
	101.4±2.6	96.5±3.4	95.5±1	96.3±3.3	98.1±1.8	99.5±1

* Values obtained by the procedure outlined in Diagram 1. Values in parenthesis are omitted from the mean.

† Single determination.

The analyses of the control solutions are quoted in full (Table 3) in order to show the amounts of N involved and to indicate the normal variation which is to be expected in replicate analyses. Replication of analyses is necessary in order to deduce a mean value because, unlike the determination of, say, total N, the highest value cannot be regarded as more probable than any other value within the normal range of recovery. The degree of accuracy of the method is not constant for all the amino-acids concerned because each acid is subject to different possible errors. It is proposed therefore to discuss the amino-acids individually, with special emphasis on the two proteins most extensively studied, insulin and edestin.

Phenylalanine. Acetylphenylalanine is the first fraction on CB₂ and its separation from the following acetylleucine(s) fraction is not easy, and mutual contamination of the two fractions may occur frequently. This is reflected in the recovery of the

two acids quoted in Table 3 in which it is clear that a low recovery of phenylalanine is usually accompanied by excessive N in the leucine fraction and vice versa. Being the first fraction, acetylphenylalanine is usually contaminated with products resulting from breakdown of the amino-acids during hydrolysis and/or acetylation, which must be eliminated by refractionation on P₅. Silica gel has been found to contain, on occasions, a gummy material which is extracted by CB₂ and which appears in the acetylphenylalanine fraction, but with care this material may be removed on P₅.

In all the control solutions studied the recovery of phenylalanine has been 98–104%, the standard deviation from the mean recoveries being 3.8% and 4% for edestin and insulin controls respectively.

Leucine isomers. The recovery of leucine(s)—unfortunately the two isomers cannot be differentiated—is, as stated above, bound up with the recovery of phenylalanine. The difficulty of effecting

Table 4. *The monoamino-acid composition of insulin, edestin, casein and β -lactoglobulin (Group 1)*

(Values quoted are N as % protein-N).

The figures in parenthesis indicate the number of hydrolyses carried out.

A. No. of acetylations (*n*).

B. Range of values where more than 3 values.

C. Mean of values with standard deviation $\sqrt{\frac{\sum d^2}{n}}$.

D. Mean recovery from appropriate control mixture.

E. Mean value after correction.

	Phenylalanine	Leucine(s)	Valine	Proline*	Tyrosine	Alanine
Insulin (5)						
A	12	13	7†	4‡	11	11
B	4.1-4.5	10.4-11.2	5.4-5.9	1.84-2.04	5.4-6.6	4.0-4.9
C	4.34±0.15	10.95±0.27	5.7±0.17	1.94±0.1	5.97±0.33	4.4±0.24
D	97.8	100	95.5	98.3	98.5	100
E	4.44	10.95	5.97	1.98	6.0	4.4
Edestin (2)						
A	7	7	6	6	6	8
B	2.3-3.1	6.1-7.3	2.84-3.2	2.1-2.7	1.8-2.0	3.4-3.9
C	2.67±0.25	6.7±0.37	2.92±0.13	2.38±0.25	1.86±0.12	3.60±0.21
D	104	94.4	94.5	92.7	96.5	98.6
E	2.6	7.1	3.1	2.6	1.9	3.65
Lactoglobulin—Hydrolysis 1 (see text)						
A	3	2	2	3	2	3
B	2.0, 2.5, 2.5	17.0, 16.7	4.0, 4.5	2.9, 2.9, 3.2	1.9, 1.9	6.0, 5.6, 6.4
C	2.33	16.85	4.25	3.0	1.9	6.0
D	102.2	98.2	95.8	c. 80	97.3	99.3
E	2.28	17.15	4.44	3.75	1.95	6.05
Lactoglobulin—Hydrolysis 2 (see text)						
A	3	3	3	3	3	3
B	2.4, 2.4, 2.25	13.4, 12.5, 13.4	4.2, 3.9, 4.5	4.1, 4.4, 4.2	1.86, 1.9, 1.83	5.7, 6.1, 6.35
C	2.35	13.1	4.2	4.23	1.86	6.03
D	102.2	98.2	95.8	99.3	97.3	99.3
E	2.30	13.3	4.4	4.25	1.91	6.07
Casein (2)						
A	4	2	4	4	4	4
B	3.35-4.0	9.7, 10.3	4.25-4.31	7.5-7.9	2.7-3.0	3.1-3.7
C	3.67	10.0	4.27	7.7	2.85	3.5
D	101.5	93.3	96.3	95	100	100
E	3.62	10.74	4.45	8.13	1.85	3.5

* Nine additional values (6.0-6.8%) rejected (see text).

† Eleven additional values (0.9-1.8) rejected (see text).

‡ See the text for a discussion of the proline values.

the separation of the two fractions is inherent in the method. It means that the application of a correction factor is somewhat arbitrary since it cannot be assumed that the contamination, if any, will be in the same direction in the analysis of the control solution as in the analysis of the protein hydrolysate. When the values are required for such a purpose as a nutritional assay this point will not be of great importance. When a higher accuracy is required for such purposes as structural analysis, it is necessary to carry out a large number of analyses so as to counterbalance the contamination as far as possible: this has been done in the case of insulin and edestin.

From Diagram 1 it can be seen that a portion of the leucine(s) is always found in the following fraction (valine, etc.). Martin & Syngé (personal communication) have ascribed this to tailing, but in the experience of the present worker the amount of leucine recovered from the valine fraction is fairly constant and is independent of the properties of the gel. It is possible therefore that factors other than tailing control this anomalous behaviour of acetyl-leucine.

Valine. The recovery of valine has been relatively constant (93-98%) in all the control mixtures studied. The main difficulty has been in the separation of valine from methionine. The methionine

content of proteins is usually low and the weak acetylmethionine band may become so diffuse and indistinct by the time it approaches the foot of the chromatogram that a separation of the fraction from valine becomes very difficult. The difficulty is enhanced by the slow rate of drainage of *cyclohexane-propanol* columns which means, almost without exception, that the fractionation has to be halted overnight. This difficulty may be overcome by using an intermediate solvent P_{10} (*cyclohexane-10% n-propanol*) when the R values are approximately twice those on P_5 . The comparative results quoted in Table 8 suggest that, in general, the valine has been estimated with a fair degree of accuracy.

Mention must be made of the valine fraction of insulin. In the early experiments on this protein, M.O. was the only available indicator. The value then quoted for valine was 6.5–6.8% (N as % total N). When ARR became available it was observed that the valine fraction was closely followed on P_5 by a second band (a band also present in acid-treated control mixtures). The artefact represented 0.5–1.0% of the total N and the valine content was found to be 5.4–5.9% of the total N, a value somewhat lower than that found by microbiological assay.

Methionine. Gordon *et al.* (1943*a*) reported that methionine could be estimated by partition chromatography and a value for the methionine content of wool was reported. The values obtained for methionine in the present work have been most disappointing. It can be seen from Table 5 that the recovery of methionine from edestin hydrolysates has been quantitative, but that low and variable recoveries have been obtained from casein and lactoglobulin hydrolysates. Similar results were obtained with the corresponding mixtures.

Table 5. *The estimation of methionine*

(Values quoted are N as % protein-N.)

	Casein	Lactoglobulin	Edestin
Found in protein hydrolysate	1.4	0.8–1.3	1.18
Recovery from control solutions (%)	66	30–64	100
Corrected value	2.1	2.0–2.6	1.18
Methionine content (Chibnall, 1946)	2.1	1.94	1.18

No definite explanation has yet been found for these vicarious recoveries, although the following observations may be noted.

(a) It is possible to recover acetylmethionine quantitatively from the chromatogram when this acid alone is subjected to the normal procedure.

(b) Acetylmethionine is recovered quantitatively from a solution of amino-acids representing the monoamino-acid fraction only of lactoglobulin.

(c) The recovery of methionine from control solutions representing the complete hydrolysate of lactoglobulin varies between 30 and 64%.

(d) When acetylmethionine equivalent to 5% of the total N was added to a lactoglobulin hydrolysate after acetylation, the recovery of methionine-N was 2.1 instead of 6.9%.

(e) These findings have been obtained with gels which had been tested by the procedure outlined above.

It has already been suggested that the amino-acid mixture and the analytical procedure may affect the recovery of the amino-acids (e.g. proline). This may be the explanation for the anomalous behaviour of methionine. So far no alternative analytical procedure has been found which would give a more satisfactory recovery. Fortunately methionine may be estimated by alternative methods and the problem is therefore not of great moment.

Proline. In the earlier experiments with insulin the variation in the proline values (0.9–1.8) obtained by the scheme of analysis (Diagram 1) was ascribed to experimental difficulties in treating the small proline fraction. Much the same view was taken of the low recoveries from the control solutions representing insulin hydrolysates. The improved recovery of proline from casein (7.9% N as % total N) and edestin (2.6%) and their attendant controls, seemed to be additional confirmation of this view. However, in experiments on lactoglobulin controls (originally designed to investigate the low recovery of methionine), it was found that, using the original procedure (Diagram 1), the recovery of proline was about 84%. When the procedure was modified to that given in Diagram 2, the recovery of proline increased to 95–103%. Similarly the changed procedure increased the proline content of lactoglobulin from about 3.0% (N as % total N) to about 4.3%. Repeat analyses have now been carried out on insulin and the corresponding control solution; the results confirm the above observation. Repeat analyses carried out on casein and edestin did not yield increased values. The obvious conclusion is that the recovery of any particular amino-acid may be affected by the composition of the mixture in which it is present, and depends to some extent upon the solvent system employed (see discussion). This point is illustrated by the observation that when ethyl acetate (Diagram 3) is used as the primary solvent, alanine and proline, instead of appearing together in the second fraction, R 0.2 (Gordon *et al.* 1943*a*), the proline is always found (at least in the amino-acid systems so far studied) in the fraction R 0.1. If, however, the alanine and glycine fractions are first

removed with CB₁₇ (i.e. Diagram 2) the proline then moves at the correct rate on Et_{ac}. Using the modified procedure (Diagram 2) the recovery of proline has been 93–99 % in all the control mixtures.

Tyrosine. The recovery of tyrosine from control solutions (Table 4) has been satisfactory. The occasional high recovery is probably due to the incomplete removal of an artefact band during the purification of tyrosine on *cyclohexane*-propanol. The recovery of tyrosine from protein hydrolysates, however, has shown wider fluctuations. This may be due to variations in the breakdown of tyrosine which is known to occur on acid hydrolysis of the protein.

Alanine. Whilst the recovery of alanine from all the control solutions studied has been satisfactory it has been necessary to exercise especial care in the separation of this amino-acid on the chromatogram. It was observed that insulin hydrolysates and the 'hydrolyzed' control solutions contained two bands *R* 0.33 and *R* 0.45 which caused considerable confusion until it was found that, with 50 % water on the gel, the *R* value of alanine was 0.45, and that this value fell to 0.35 (the normal value reported by Gordon *et al.* 1943*a*) when the water content was increased to, say, 70 %. For this reason it is recommended that the *R* value of acetylalanine be checked for each sample of gel used. (This will be done as part of the routine test on silica gel suggested above.) The possible occurrence of a similar artefact in other hydrolysates should always be borne in mind.

In the above discussion no mention has been made of the occasional value which was obviously fortuitously high or low. In the three years in which this method has been in use in this laboratory, such results have been obtained when there was no reason to suspect any experimental error. It is therefore essential that all analyses should be done at least in triplicate and that each estimation should be based on an independent acetylation.

lysates. These findings cannot be due to simple tailing, because, in the case of methionine, the lost N cannot be detected in any other fraction and in the instance of proline on ethyl acetate the *R* value of the entire fraction has been changed. So far as is known the anomalous behaviours of proline and methionine are independent of the silica gel and it has been observed in preliminary tests on gels prepared by Isherwood (1946) that the behaviour of proline is similar to that reported above. These two amino-acids are the only ones that have been found, so far, to give anomalous results, but the observation emphasizes the necessity for using *ad hoc* control mixtures in all protein analyses where high accuracy is desired, especially when the composition of the protein is abnormal.

One further observation is worthy of note. It has been the usual practice to check the titration of the acetamino-acids by estimating N in the neutralized solution in order to detect the presence of organic acids, which may arise either from decomposition of the protein itself during hydrolysis, or from impurities present in the original protein preparation. It has been observed, however, that on occasions, even in the analysis of *ad hoc* control mixtures, the two values showed divergence. Usually the Kjeldahl value was higher than the carboxyl titration. A possible explanation is that the indicators are leached and that in the case of ARR, which is an ammonium salt, hydrolysis has occurred with the liberation of ammonia. In the instances in the present work where such a difference has occurred it has been customary to accept the carboxyl titration provisionally and to repeat the estimation.

DISCUSSION

Gordon *et al.* (1943*a*) suggested that the chromatographic method should give values with an accuracy of 5 %, and added that this accuracy might be increased by the use of *ad hoc* control mixtures.

Table 6. *The monoamino-acid composition of various proteins (Group 2)*

(Values quoted are N as % protein-N.)						
No. of analyses	Standard correction (see Table 3)	Zein	Gliadin	Ovalbumin	Gelatin	Collagen
Phenylalanine	101.4	3	2	4	2	2
Leucine	96.5	3.1	3.1	4.0	1.2	1.9
Isoleucine		14.8	7.2	10.8	3.1	3.2
Valine	95.5	2.6	1.8	3.5	2.5	2.2
Proline	96.3	7.9	9.2	2.5	10.0	9.9
Tyrosine	98.1	1.9	1.4	1.9	0.5	0.6
Alanine	99.5	10.2	1.9	6.7	8.1	8.0

Anomalous findings. Attention has already been called to the behaviour of proline and to the inability to recover methionine from certain hydro-

The desired accuracy will, of course, depend upon the purpose of the analysis. Thus for nutritional assay an overall accuracy of 10 % will probably

suffice and it will therefore be unnecessary to carry out rigid control experiments. When, however, the values are required for purposes such as the estimation of the number of residues of the amino-acid(s) in the minimum molecule, it is clear that a higher order of accuracy will be necessary. The required limits of accuracy, which will depend upon the number of residues of any particular amino-acid in the minimum molecule, are illustrated in Table 7. For the purposes of calculation it has been assumed that, if n is the number of residues of the amino-acid in question, then the maximum permitted variation in the values will be $\pm 0.4/n$. For example, the minimum molecule of insulin (12,000) contains six residues of phenylalanine

recovery of the amino-acids from control solutions representing hydrolysates of this protein was satisfactory.

From Table 7 it is clear that, in the case of insulin, the analysis of the control mixture has given recoveries within the limits defined above (i.e. $n \pm 0.4/n$) and that this accuracy has also been realized in the analysis of the protein hydrolysate. In this case therefore the application of the correction factors to the mean values obtained for the various amino-acids appears to be fully justified. In the analysis of the control solutions representing edestin a similar degree of accuracy has been observed, but it is clear that, with the general increase in the number of residues in the

Table 7. *The accuracy required in amino-acid analysis when estimating the number of residues in the protein (sub-) molecule*

- (1) Assumed number of residues (Chibnall, 1946).
- (2) Required accuracy (%), see text.
- (3) Accuracy observed in control experiments (%).
- (4) Accuracy observed in protein analyses (%).

	Insulin				Edestin			
	(1)	(2)	(3)	(4)	(1)	(2)	(3)	(4)
Phenylalanine	6	6.7	4	3.4	18	2	4	10
Leucine(s)	14	3	4	2.7	48	1	4	6
Valine	8	5.0	2.5	3.0	21	2	4	3
Proline	3	13	3	5.5	16	2.5	1.5	11
Alanine	6	7	4	5.5	24	1.5	<1	5.5

(Chibnall, 1946) and the values will thus have to be within $\pm 6.7\%$ ($\pm 0.4/6$) of the actual value if the number of residues is to be put at 6 and not 5 or 7.

Evidence obtained in the present work suggests that the precision is very dependent upon the properties of the silica gel in use. Even with an efficient gel occasional fortuitously high or low values may be obtained and the recovery of acetamino-acids from the control solutions may not show close duplication. For this reason it is considered that a single control experiment cannot be used to correct single values obtained simultaneously on the protein hydrolysate, but that, as far as possible, the statistical approach should be adopted. In this way it is possible to eliminate any such fortuitous results as may be obtained and to deduce the mean amino-acid content of the protein hydrolysate. It is clear from the literature that this procedure has been adopted by the more critical workers in the microbiological assay of the amino-acids (cf. Table 4; Dunn, Camien, Shankman, Frankel & Rockland, 1944; Ryan & Brand, 1944).

It is not proposed to include casein and β -lactoglobulin in the discussion of the probable accuracy of the method because the values obtained for the former protein are few in number. In the latter case certain difficulties were encountered in the analysis of proline and leucine, although the

edestin submolecule, this accuracy is not generally satisfactory. With hydrolysates of edestin, values have shown variations of as much as 10%. In this case two factors may be concerned:

(a) Most of the analyses on insulin and its control solutions were carried out in the early stages of this work. The samples of silica then being used were very satisfactory, and their preparation was a routine matter. More recently, however, the preparation of silica gel with satisfactory properties has become difficult and it is now apparent that gels which appeared at the time to be satisfactory by the phenylalanine-leucine test were actually adsorbing the more hydrophilic acetamino-acids. Unfortunately certain of the edestin values were obtained on gels which may have been partially adsorbent, nevertheless all values have been included in the calculation of the mean in order not to suggest what might be a fictitiously high degree of accuracy.

(b) It has been observed that the recovery of amino-acids from simple control solutions is less erratic than from solutions representing complete protein hydrolysates, particularly when tryptophan was present. (Insulin does not contain tryptophan, methionine or hydroxyproline.)

So far the accuracy of the method has been judged by the analysis of control solutions representing the various proteins under examination. This procedure, whilst enabling an estimation to be made of hydrolytic (other than those of fissional origin) and manipulative losses, cannot show up any

inherent error in the method of analysis itself. Thus if the apparent quantitative recovery of the various fractions is the result of a cancellation of errors, then although both protein hydrolysate and control solution will be equally affected, the method cannot be regarded as fully satisfactory, particularly if a second method is available which is not so affected.

A second method of approach is to compare the values obtained for any well-characterized protein with those obtained by other methods, preferably those which make use of entirely different principles of analysis. This aspect of the present work has taken on added importance with the intense development of the microbiological assay of amino-acids. Apart from isotope dilution, which has only been applied in very few instances, and which, by virtue of requiring the isolation of a pure sample of the amino-acid (or of a derivative), perpetuates some of the difficulties of the older gravimetric methods, it is the only reasonably satisfactory procedure with which to compare the microbiological assay of the simple monoamino-acids. The value of the comparative approach was realized by Ryan & Brand (1944) who stated that 'In a conservative approach we would refrain from considering such microbiological values as significant without other confirmations such as comparisons with dependable data in the literature'.

This point has been illustrated by Chibnall (1946), who compared the glutamic acid content of various proteins as obtained by gravimetric analysis and microbiological assay, and showed that when the values obtained by the latter procedure were low, this might be explained by the presence of *d*(-)-glutamic acid in the protein hydrolysate.

In general the values obtained by microbiological assay and partition chromatography are in close agreement (Table 8), although comparison is still limited to a few proteins. It is nevertheless not yet possible to regard all the values as final because certain of those obtained in the present work have no doubt been affected by the unwitting use of unsatisfactory samples of silica gel. Similarly, the variations in the microbiological values suggest that the method is still in the process of development and that it is not yet possible to define the limits of its accuracy.

If protein hydrolysates, but not the 'hydrolyzed' control mixtures, contain artefacts which affect the method of analysis, then the application of control recoveries to the protein hydrolysate is not strictly valid. Many of the errors encountered in the present work are no doubt due to the presence of such artefacts of hydrolysis, and there is every reason to suppose that other methods of analysis, such as microbiological assay, will also be affected. Indeed the values so far obtained by microbiological assay

certainly suggest that the method is subject to errors of this kind. In fact these values indicate that, whatever the ultimate advantage, the microbiological is at present no more precise than partition chromatography. Thus Dunn, Camien, Shankman *et al.* (1944) who, incidentally, unlike many workers in this field, have used *ad hoc* control mixtures to increase the precision of the assay, reported that in the assay of lysine (by means of *Leuconostoc mesenteroides* P₆₀) in control mixtures the recoveries varied between 87.7 and 114.0%, although the mean of 16 values was 100.6%. Similarly the assay of lysine in casein hydrolysates gave values ranging between 6.93 and 9.0%. In the latter case the mean was unaffected by the omission of the extreme values. Table 7 also includes some values obtained by chemical methods. Whilst certain of the leucine, isoleucine and valine values are in reasonable agreement with chromatographic and microbiological values, they carry such large corrections that the methods (cf. Block & Bolling, 1945*b*; Mourgue, 1943) must be considered as unsatisfactory.

Attention should be called to two of the values reported in Table 8. (1) The chromatographic values for the leucine-isoleucine content of lactoglobulin are 17.1 and 13.3% (N as % total N). The latter value was obtained in triplicate on a second hydrolysate and it is thought that the low value was due to incomplete hydrolysis. Shortage of lactoglobulin has made it impossible to repeat the analysis. (2) The total leucine isomers found for insulin by both methods is 10.8% (N as % total N), but the distribution of leucine and isoleucine is 8.9/1.9 (microbiological) and 7.8/3.2 (chromatographic separation coupled with the use of infra-red analysis). The probable accuracy of infra-red analysis will be discussed in a publication dealing with the application of the method, but Dr G. B. B. M. Sutherland (private communication) states that the above figure is probably accurate to $\pm 5\%$.

The unsatisfactory nature of casein as a control protein (stressed by Chibnall, 1946) is shown in Table 8 by the variations in the values found by microbiological assay. The suggestion is put forward that in the development of accurate methods of protein analysis, it would be preferable to use products known to be chemical individuals (e.g. insulin and β -lactoglobulin).

The chromatographic technique is considered by the present worker to be potentially capable of high accuracy. At present it suffers from several handicaps which are not only reducing the precision but making the method laborious because of the necessity of carrying out replicate analyses. In addition, unaccountable false values are found which tend to increase the uncertainty in the method. The solution to the problem is chiefly

Table 8. *Comparison of methods for the estimation of monoamino-acids in proteins.*
(Taken in part from Chibnall (1946))

(Values quoted are N as % protein-N.)

Protein	Amino-acid	Partition chromatography	Microbiological assay	Chemical methods
Casein	Valine	4.45 ⁽¹⁾	5.1 ⁽²⁾ , 3.7 ⁽³⁾ , 5.1 ⁽⁴⁾ , 5.6 ⁽⁴⁾ , 4.7 ⁽⁵⁾ , 5.1 ⁽⁶⁾ , 4.85 ⁽¹⁶⁾	3.45 ⁽¹⁹⁾
	Phenylalanine	3.62 ⁽¹⁾	2.0 ⁽³⁾ , 3.2 ⁽⁶⁾ , 2.66 ⁽¹⁷⁾	2.77 ⁽¹²⁾ , 3.35 ⁽¹⁸⁾ , 2.55 ⁽¹⁸⁾
	Leucine	7.2 ⁽¹⁾	6.7 ⁽⁷⁾ , 6.2 ⁽⁴⁾ , 6.3 ⁽⁸⁾ , 8.0 ⁽⁹⁾ , 6.8 ⁽⁶⁾ , 6.7 ⁽¹⁵⁾ , 6.5 ⁽¹⁶⁾	11.1 ⁽¹⁹⁾
	Isoleucine*	3.6 ⁽¹⁾	3.8 ⁽⁸⁾ , 4.2 ⁽⁴⁾ , 4.1 ⁽⁸⁾ , 4.3 ⁽⁹⁾ , 4.1 ⁽¹⁶⁾	
	Alanine	3.5 ⁽¹⁾		5.6 ⁽¹²⁾
β -Lactoglobulin	Alanine	6.1 ⁽¹⁾	6.25 ⁽¹⁰⁾	
	Valine	4.45 ⁽¹⁾	4.47 ⁽¹⁰⁾ , 4.21 ⁽⁶⁾ , 4.44 ⁽²⁾	5.9 $\pm$ 1 ⁽¹⁸⁾
	Phenylalanine	2.3 ⁽¹⁾	1.93 ⁽¹⁰⁾ , 2.33 ⁽⁶⁾	2.8 ⁽¹⁸⁾
	Leucine	17.1 ⁽¹⁾ (13.3) ⁽¹⁾	10.68 ⁽¹⁰⁾ , 10.47 ⁽⁶⁾	11.7 $\pm$ 2.8 ⁽¹⁸⁾
	Isoleucine		5.75 ⁽¹⁰⁾ , 4.79 ⁽⁶⁾	4.4 $\pm$ 0.4 ⁽¹⁸⁾
	Proline	4.3 ⁽¹⁾	3.2 ⁽¹⁰⁾	
Ovalbumin	Valine	3.5 ⁽¹⁾	5.2 ⁽²⁾ , 5.3 ⁽⁶⁾	2.9 ⁽¹⁹⁾
	Phenylalanine	4.0 ⁽¹⁾	4.2 ⁽⁶⁾	2.9 ⁽¹²⁾ , 4.2 ⁽¹⁸⁾
	Leucine	10.8 ⁽¹⁾	6.6 ⁽⁷⁾ , 6.2 ⁽⁶⁾ , 6.6 ⁽¹⁵⁾	9.4 ⁽¹⁹⁾
	Isoleucine		4.74 ⁽⁶⁾	
	Alanine	6.7 ⁽¹⁾		7.2 ⁽¹²⁾
Edestin	Valine	3.1 ⁽¹⁾	3.0 ⁽³⁾	4.1 ⁽¹⁹⁾
	Leucine	5.0 ⁽¹⁾	5.1 ⁽³⁾ , 5.6 ⁽³⁾	3.75 ⁽¹⁹⁾
	Isoleucine*	2.1 ⁽¹⁾		
	Phenylalanine	2.6 ⁽¹⁾	1.9 ⁽³⁾	
Insulin	Valine	6.0 ⁽¹⁾	6.6 ⁽¹¹⁾	
	Phenylalanine	4.4 ⁽¹⁾	4.2 ⁽¹¹⁾	4.45 ⁽¹⁸⁾
	Leucine	7.8 ⁽¹⁾	8.9 ⁽¹¹⁾	
	Isoleucine*	3.2 ⁽¹⁾	1.9 ⁽¹¹⁾	
	Proline	2.0 ⁽¹⁾	2.2 ⁽¹¹⁾	
Gelatin	Phenylalanine	1.2 ⁽¹⁾ , 0.4 ⁽¹³⁾ , 1.0 ⁽¹⁴⁾	1.1 ⁽⁶⁾ , 1.1 ⁽¹⁶⁾	1.0-1.2 ⁽¹⁸⁾
	Leucine	3.1 ⁽¹⁾ , 3.9 ⁽¹³⁾ , 3.9 ⁽¹⁴⁾	1.95 ⁽⁸⁾ , 2.1 ⁽⁶⁾ , 2.2 ⁽⁷⁾ , 2.13 ⁽¹⁵⁾ , 1.94 ⁽¹⁶⁾	2.5 $\pm$ 0.35 ⁽¹⁸⁾ , 4.1 ⁽¹⁹⁾
	Isoleucine		1.01 ⁽⁸⁾ , 1.02 ⁽⁶⁾ , 0.85 ⁽¹⁶⁾	0.73 $\pm$ 0.13 ⁽¹⁸⁾
	Valine	2.5 ⁽¹⁾ , 1.7 ⁽¹³⁾ , 1.5 ⁽¹⁴⁾	1.6 ⁽⁸⁾ , 1.8 ⁽⁶⁾ , 1.8 ⁽²⁾ , 1.7 ⁽¹⁶⁾	1.6 $\pm$ 0.75 ⁽¹⁸⁾ , 0.9 ⁽¹⁹⁾
	Proline	10.0 ⁽¹⁾ , 9.6 ⁽¹³⁾ , 9.7 ⁽¹⁴⁾		
	Alanine	8.1 ⁽¹⁾ , 8.3 ⁽¹³⁾ , 9.0 ⁽¹⁴⁾		
Zein	Leucine	14.8 ⁽¹⁾	10.3 ⁽¹⁵⁾	17.0 $\pm$ 0.7 ⁽¹⁸⁾
	Isoleucine			2.8 $\pm$ 0.2 ⁽¹⁸⁾
	Valine	2.6 ⁽¹⁾		2.1 $\pm$ 0.7 ⁽¹⁸⁾

* Leucine-isoleucine ratios determined by Dr G. B. B. M. Sutherland (private communication).

- (1) Present work.
- (2) McMahan & Snell (1944).
- (3) Hegsted (1944).
- (4) Dunn, Camien, Stockland, Shankman & Goldberg (1944).
- (5) Schweigert (1945).
- (6) Stokes, Gunness, Dwyer & Caswell (1945).
- (7) Ryan & Brand (1944).
- (8) Kuiken, Norman, Lyman, Hale & Blotter (1943).
- (9) Block & Bolling (1945*a*).

- (10) Brand, Ryan & Diskant (1945).
- (11) Brand (private communication).
- (12) Virtanen, Laine & Toivonen (1940).
- (13) Gordon *et al.* (1943*a*).
- (14) Gordon *et al.* (1943*b*).
- (15) Brand *et al.* (1945).
- (16) Hier, Graham, Friedes & Klein (1945).
- (17) Dunn, Shankman & Camien (1945).
- (18) Block & Bolling (1945*b*).
- (19) Mourgue (1943).

bound up with the preparation of silica which has a high standard of efficiency. This now seems to be possible, especially if the apparent effect of water (in the absence of iron) is borne out by experience.

A further improvement might be effected if the method could be used in conjunction with a procedure, say electrodialysis, which would yield the monoamino-acids quantitatively and relatively free from other amino-acids. The reason for this suggestion is the repeated observation that mixtures

limited to monoamino-acids give much more satisfactory recoveries than those containing the full range of amino-acids found in protein hydrolysates.

SUMMARY

1. Partition chromatography has been extensively applied to the determination of the monoamino-acids in protein hydrolysates.

2. Certain difficulties were encountered in the application of the original analytical procedure (Gordon *et al.* 1943*a*). A modified procedure is suggested which has been found satisfactory in the amino-acid systems studied.

3. The important factors in the preparation of silica gel have been discussed and evidence presented which suggests that, for iron-free gels, the adsorption of acetamino-acids by silica gel is dependent upon the water content of the silica.

4. The values obtained for insulin, edestin, casein and β -lactoglobulin have been submitted to control by the analysis of the corresponding *ad hoc* mixtures of amino-acids. The probable accuracy of the method is discussed with special reference to insulin and edestin.

5. The values reported for zein, gliadin, ovalbumin, gelatin and collagen are regarded as

provisional only, because they have not been submitted to control by the analysis of *ad hoc* mixtures of amino-acids.

6. The values obtained have been compared with those found by other methods of analysis, especially the values obtained by microbiological assay.

7. It is suggested that, in the development of accurate methods of analysis, it would be preferable to use products known to be chemical individuals (e.g. insulin and β -lactoglobulin) as the test proteins.

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